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### (54) MONOCLONAL ANTIBODY THAT INDUCES APOPTOSIS

(57) A monoclonal antibody that induces apoptosis in the myelocytic cell, a fragment of the antibody, and a hybridoma that produces the antibody. The antibody is useful as an antibody that specifically recognizes an antigen or the like inducing apoptosis in the myelocytic cell and discriminates and identifies the same. Further it is useable as a useful drug in the field of, for example, treating myelocytic leukemia by utilizing the activity of inducing apoptosis in the myelocytic cell.

**Description****Technical Field**

The present invention relates to a novel monoclonal antibody having the property of causing apoptosis on myeloid cells and being useful as medicine for myelocytic leukemia, and fragments thereof, and furthermore, relates to a hybridoma producing the monoclonal antibody.

Since the monoclonal antibodies of the present invention are useful as antibodies recognizing and identifying antigens causing apoptosis on myeloid cells specifically and besides have the property of causing apoptosis on myeloid cells, they may be used as medicine useful in the field of remedies for myelocytic leukemia utilizing the property.

**Background Art**

Granulocyte colony-stimulating factors, for example, recombinant granulocyte colony-stimulating factors (rG-CSF), have been known primarily as humoral factors to stimulate the differentiation and proliferation of granulocyte cells, and it has been reported in an experiment upon mice *in vivo* that the administration of rG-CSF enhances the hematopoiesis of the bone marrow and in addition causes remarkable extramedullary hematopoiesis in the spleen to proliferate hematopoietic stem cells and all hematopoietic precursor cells in the spleen. And it has been thought as extramedullary hematopoietic mechanism in the spleen that hematopoiesis occurs due to a splenic hematopoietic microenvironment modifications according to the stimulation of rG-CSF to enhance hematopoietic potential.

Hence, the present inventors have noted splenic stromal cells administered rG-CSF with a view to clarifying the hematopoietic potential in the spleen, and established a hematopoietic stromal cell line (CF-1 cells) from the spleen of a mouse administered rG-CSF with a view to attempting the analysis of the enhancement of the hematopoietic potential by stromal cells with rG-CSF, and examined the potential effect on hematopoiesis using the hematopoietic stromal cells, and as a result, colony-stimulating activities *in vitro* and potency supportive of hematopoietic stem cells *in vivo* have been recognized [Blood, 80, 1914 (1992)].

However, though some of splenic stromal cells have been established as a cell line (CF-1 cells), and cytological characteristics thereof have been examined, no specific antibody recognizing surface antigens thereof has been prepared so far, and characteristics thereof have been scarcely known yet.

Hence, the present inventors have engaged in assiduous studies with a view to developing specific antibodies capable of recognizing splenic stromal cells on the basis of the above information upon splenic stromal cells and the results of the studies, and prepared monoclonal antibodies using the splenic stromal cell lines as anti-

gens for immunization, and as a result, novel monoclonal antibodies unreported so far have been obtained.

And as a result of examining the properties of the obtained monoclonal antibodies, the inventors have found surprisingly that they have the property of causing apoptosis on myeloid cells, which has led to the completion of the present invention.

**Disclosure of Invention**

It is the objective and purpose of the present invention to provide a novel monoclonal antibody having the property of causing apoptosis on myeloid cells and being useful as medicine for myelocytic leukemia, and fragments thereof, and in addition a hybridoma producing the monoclonal antibody.

The monoclonal antibody of the present invention is remarkably useful as an antibody recognizing antigens causing the apoptosis [ it is also called self-destruction of cells, phenomenon that a nuclear chromatin DNA is digested at a nucleosome unit (so-called ladder formation) to result in the death of cells ] of myeloid cells and having a function of identifying them or a function of causing apoptosis on myeloid cells. Incidentally, myeloid cells include cells other than lymphoid cells, such as neutrophils, megakaryocytes, myeloblasts, myelocytes, mast cells, macrophages, monocytes and erythroblasts, and the myeloid cells according to the present invention also mean the same as mentioned above. No monoclonal antibody having the property of causing apoptosis on myeloid cells has been known so far, and hence the monoclonal antibodies of the present invention are defined to include all monoclonal antibodies having the property of causing apoptosis on myeloid cells.

The monoclonal antibody of the present invention may be prepared basically as stated below.

Namely, the monoclonal antibody of the present invention may be prepared, for example, by using splenic stromal cells derived from an animal administered rG-CSF as antigens, immunizing them according to an ordinary immunization method, cell-fusing the immunized cells according to an ordinary cell fusion method, and cloning the fused cells according to an ordinary cloning method.

As a method of preparing the monoclonal antibody of the present invention can be preferably exemplified a method comprising using CF-1 cells, splenic stromal cells of an animal administered rG-CSF established as culture cell line by the present inventors, as the antigen [Blood, Vol. 80, 1914 (1992)], fusing plasma cells (immunocyte) of a mammal immunized with the antigen with myeloma cells of a mammal such as a mouse, cloning the obtained fused cells (hybridomas), selecting clones producing antibody according to the present invention recognizing the above cell line among them, and culturing them to recover objective antibody. However, the method is only an example, and in this case, for example, not only the above CF-1 cells but also cell lines derived from human splenic stromal cells obtained according to

the case of CF-1 cells may be used as the antigens properly to prepare antibodies binding to objective human myeloid cells in the same manner as in the case of the above CF-1 cells.

In the method of preparing such monoclonal antibodies, mammals to be immunized with the above antigen are not particularly restricted; it is preferable to make selection taking into account suitability with myeloma cells to be used in cell fusion, and preferably a mouse, a rat and a hamster are used.

Immunization is performed according to an ordinary method, for example, by administering splenic stromal cells such as the above CF-1 cells into abdominal cavity of a mammal by injection. More specifically, it is preferable to administer one diluted with or suspended in a proper amount of PBS or isotonic sodium chloride solution to an animal several times every month. It is preferable to use splenic cells removed after the final administration of the above cells as immunocytes.

As a myeloma cell of a mammal as the other parent cell fused with the above immunocytes can be used preferably known various cells including P3(P3X63Ag8.653) [J. Immunol., 123, 1548 (1978)] , p3-U1 [Current Topics in Micro-biology and Immunology, 81, 1-7 (1978)] , NS-1 [Eur. J. Immunol., 6, 511-519 (1976)] , MPC-11 [Cell, 8, 405-415 (1976)] , Sp2/0-Ag14 [Nature, 276, 269-270 (1978)] , FO [J. Immunol. Meth., 35, 1-21 (1980)] , S194 [J. Exp. Med., 148, 313-323 (1978)] and R210 [Nature, 277, 131-133 (1979)].

The cell fusion of the above immunocyte and a myeloma cell may be performed basically according to an ordinary method, for example, a method by Milstein et al. [Methods Enzymol., 73, 3-46 (1981)].

More specifically, the above cell fusion may be performed, for example, in an ordinary nutrition medium in the presence of a fusion-accelerating agent. As a fusion-accelerating agent, polyethylene glycol (PEG) and Sendai virus (HVJ), and furthermore, adjuvants such as dimethyl sulfoxide may be added properly if required in order to enhance the fusing effect. Regarding the ratios of immunocytes and myeloma cells, the former is preferably used in an amount 1-10 times that of the latter. Examples of a medium used in the above cell fusion include a RPMI-1640 medium and a MEM medium suitable for the proliferation of the above myeloma cell and other mediums ordinarily used for the culture of this kind of cell, and in addition, supplementary serum such as fetal bovine serum (FBS) may be used together.

Cell fusion is performed by mixing prescribed amounts of the above immunocytes and myeloma cells in the above medium, adding a PEG solution preheated to about 37°C, for example, PEG with an average molecular weight of the order of 1,000-6,000 to the medium, ordinarily, at a concentration of about 30-60 % (W/V), and mixing them. Subsequently, by repeating the operations of adding proper mediums one after another, centrifuging the reaction mixture and removing the supernatants can be formed objective hybridomas.

Said hybridomas are selected by culturing in an ordinary selective medium, for example, a HAT medium (medium supplemented with hypoxanthine, aminopterin and thymidine). The culture in the HAT medium is continued for a period sufficient for cells other than objective hybridomas (non-fused cells) to die out, ordinarily for several days to several weeks. Subsequently, the screening and monocloning of the hybridomas producing the objective antibodies are performed according to ordinary limiting dilution analysis.

The prepared hybridomas producing the monoclonal antibodies of the present invention may be subcultured in an ordinary medium and stored in liquid nitrogen for a long time.

In order to collect the monoclonal antibodies of the present invention from the hybridomas may be employed a method comprising culturing the hybridomas according to an ordinary method, and obtaining them from the supernatants, or a method comprising administering a hybridoma into a appropriate mammal to proliferate, and obtaining them from its ascite. The former is suitable for obtaining antibodies with a high purity and the latter is suitable for the mass production of antibodies.

Furthermore, the antibodies obtained according to the above methods may be purified to a high degree employing an ordinary purification means such as a salting-out technique, gel filtration and affinity chromatography.

The monoclonal antibody of the present invention may be any one so far as it has a specific property to be described specifically in Example later, namely, a property of causing apoptosis on myeloid cells, and those having the property are included in the scope of the present invention, irrespective of the kind of antigens; the monoclonal antibody of the present invention may be used as useful medicine for myelocytic leukemia according to utilizing the property.

Needless to say, the establishment of a specific system for identifying and recognizing antigens causing apoptosis on myeloid cells according to utilizing the monoclonal antibody of the present invention, or for using it as medicine for myelocytic leukemia according to utilizing the specific property thereof, and modification and application thereof are included within the scope of the present invention so far as they are put into practice according to an ordinary method obvious to those skilled in the art.

#### Brief Description of Drawings

Fig. 1 shows an analysis (a control in the absence of an antibody, CF-1 cell) according to immunofluorescence.

Fig. 2 shows an analysis of the binding properties of the GSPST-1 antibody to CF-1 cells according to immunofluorescence.

Fig. 3 shows an analysis of the binding properties of the BMAP-1 antibody to CF-1 cells according to immunofluorescence.

Fig. 4 shows an analysis (a control in the absence of an antibody, bone marrow cell) according to immunofluorescence.

Fig. 5 shows an analysis of the binding properties of the GSPST-1 antibody to bone marrow cells according to immunofluorescence. 5

Fig. 6 shows an analysis of the binding properties of the BMAP-1 antibody to bone marrow cells according to immunofluorescence.

Fig. 7 shows an analysis (a control in the absence of an antibody, NFS-60) according to immunofluorescence. 10

Fig. 8 shows the binding properties of the GSPST-1 antibody to NFS-60 cells according to immunofluorescence. 15

Fig. 9 shows an analysis (a control according to rat IgG1, NFS-60) according to immunofluorescence.

Fig. 10 shows the binding properties of the BMAP-1 antibody to NFS-60 cells according to immunofluorescence. 20

Fig. 11 shows an assay for the monoclonal antibody (BMAP-1) to inhibit NFS-60 cell proliferation.

Fig. 12 shows an assay for the monoclonal antibody (GSPST-1) to inhibit the bone marrow transplantation.

Fig. 13 shows an assay for the monoclonal antibody (BMAP-1) to inhibit the bone marrow transplantation.

Fig. 14 is an explanatory view [microphotograph (stained with H. E.) of bone marrow samples ( $\times 400$ )] showing dead bone marrow cells (2) on 6 days after the administration of the monoclonal antibody BMAP-1 of the present invention, and the control (1) in the absence of the antibody. 25

Fig. 15 is an explanatory view (migration-photo according to electrophoretic chromatography) showing the ladder formation of the DNA of bone marrow cells observed when the monoclonal antibody BMAP-1 of the present invention was administered.

Fig. 16 shows a cytotoxicity assay using L929 cells by TNF  $\alpha$ .

Fig. 17 shows a cytotoxicity assay by the monoclonal antibody (BMAP-1).

Fig. 18 shows an analysis (a control according to rat IgG2a, BWV1) according to immunofluorescence.

Fig. 19 shows the binding properties of the anti-mouse MHC class I antibody to BWV1 cells according to immunofluorescence. 45

Fig. 20 shows an analysis (a control according to rat IgG1, BWV1) according to immunofluorescence.

Fig. 21 shows the binding properties of the BMAP-1 antibody to BWV1 cells according to immunofluorescence. 50

#### Explanation of Symbols

a: DNA of the thymus of a mouse administered BMAP-1 (24 hours)

b: DNA of the bone marrow of a mouse administered BMAP-1 (24 hours)

c: DNA of the bone marrow of a mouse administered BMAP-1 (8 hours)

d: DNA of the bone marrow of a mouse administered BMAP-1 (4 hours)

e: DNA of the bone marrow of a non-treated mouse (bone marrow cells)

f: Molecular weight marker

Next, the present invention will be described further in detail according to Reference Example and Example, but the present invention is not restricted to the Example.

#### Reference Example

##### Establishment of Splenic Stromal Cells and Their Characteristics Thereof

###### 1) Establishment of Splenic Stromal Cells

A splenic stromal cell line was established from the primary culture of the splenic cells of a C57BL/6J mouse administered rG-CSF 100  $\mu$ g/kg for 5 days. Namely, this spleen was removed after the administration of rG-CSF under germ-free conditions, cultured in a 25-cm<sup>2</sup> plastic flask (Corning Co.) for 6 weeks and in an Isocove's modified Dulbecco's medium (IMDM) (Boehringer-Mannheim Co.) with 10 % heat-inactivated fetal bovine serum (FBS) (Sanko Junyaku, Tokyo), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin in an incubator under the condition of 37 °C and 5 % CO<sub>2</sub>, and the medium was exchanged for a fresh growth medium twice a week.

In the confluent culture, the adherent cell populations (stromal cells) were harvested from the flask by using 0.05 % trypsin plus 0.02 % EDTA (Sigma Chemical Co.) in Ca-,Mg-free PBS, and were transferred into new flasks. These passages were repeated approximately once or twice a week. In the early passages (1st through 10th passages), the split ratio of the cells was 1/4 to 1/8, and subsequently the ratio was 1/16 to 1/32. The stromal cells became homogeneous and fibroblastoid after approximately the 10th passage.

At the 20th passage, these stromal cells were harvested as described above and forwarded to cell cloning by using a limiting dilution technique; cell cloning was repeated twice to establish a stromal cell line (CF-1 cell line).

Subsequently, these cells were maintained in 5 ml of IMDM supplemented with 10 % heat-inactivated FBS in a 25-cm<sup>2</sup> flask (Corning Co.), and subcultured once every 5 days at the split ratio of 1/32. Splenic stromal cell lines can be established from other animals than mouse; for example, human splenic stromal cell lines can be established using the same method as described above by transforming the cells with an SV-40 adenovirus vector [J. Cell. Physiol., 148, 245 (1991)].

## 2) Characteristics of CF-1 Cells

CF-1 cells established as a cell line as described above were examined for alkaline phosphatase, acid phosphatase,  $\beta$ -glucuronidase,  $\alpha$ -naphthyl acetate esterase and oil red O using standard cytochemical techniques. CF-1 cells were also characterized by immunoenzymatic histochemistry using the following monoclonal and polyclonal antibodies: macI (Sero Tec.); factor VIII-related antigen (Dakopatts); and collagen type I, collagen type III and fibronectin (Chemicon International Inc.). Phagocytosis was tested by latex bead uptake (particle diameter: 1.09  $\mu\text{m}$ ; Sigma), and the potency of CF-1 cells to convert to adipocytes was tested by exposure to  $10^{-6}$  mol/l hydrocortisone phosphate (Sigma) in a 25-cm<sup>2</sup> flask for 4 weeks after the confluent culture.

As a result, the CF-1 cells were negative for alkaline phosphatase, factor VIII-related antigen, mac I and phagocytosis, whereas they were positive for collagen type I, collagen type III and fibronectin. CF-1 cells were not converted to adipocytes during 4 weeks in a confluent culture with  $10^{-6}$  mol/l hydrocortisone, although CF-1 cells had only traces of lipid. From these data, CF-1 cells do not have the characteristics of preadipocytes, macrophages and endothelial cells, and therefore it has become obvious that they are derived from stromal cells different from them.

## 3) Maintenance of Hematopoietic Stem Cells by CF-1 Cells

To examine whether hematopoietic stem cells are maintained by CF-1 cells or not, CFU-S assays (assays of spleen colonies-forming cells) were performed by the technique of Till and McCulloch. Ten mice per group were irradiated with 900 cGy (MBR-1520R; Hitachi, Tokyo) and injected intravenously with bone marrow mononuclear cells (BM cells) ( $1.0 \times 10^5$ /head,  $5.0 \times 10^4$ /head, or  $2.5 \times 10^4$ /head) and CF-1 cells ( $1.0 \times 10^5$ /head), and colonies in the spleen were counted on the 12th day as CFU-S clones (spleen colonies).

As a result, when bone marrow mononuclear cells (BM cells) and CF-1 cells were transplanted into irradiated mice, the number of spleen colonies of every group of BM cells increased significantly (between 1.4-1.8 times) as compared to the mice without CF-1 cells, and, on the 12th day after the transplantation, the survival ratios of the mice transplanted with BM cells and CF-1 cells were higher than those with only BM cells, showing a low death rate; hence it has become apparent that hematopoietic stem cells are maintained by CF-1 cells.

## Best Mode for Carrying Out the Invention

The embodiment of the invention will be described in detail hereinafter.

## Example

### Establishment of Monoclonal Antibodies

#### 5) 1) Antigens and Immunization

Immunization was performed by using CF-1 cells obtained in the above Reference Example as antigens. The cells were cultured in an incubator under the condition of 5 % CO<sub>2</sub> and 37 °C, using an Isocove's modified Dulbecco's medium (IMDM) (Boehringer-Mannheim Co.) supplemented with 10 % fetal bovine serum (FBS; Sanko Junyaku) as a medium.

The cells were treated with 1mM EDTA/PBS, and removed from a culture flask according to pipetting. The cells were suspended into 1mM EDTA/PBS at the cell number of about  $1 \times 10^7$ /ml, and administered to a Wistar Imamich rat (7-week-old, female; Animal Breeding Research Laboratory). One ml of cells of about  $1 \times 10^7$ /ml were injected into the abdominal cavity of the rat at the initial immunization, and 1 ml of cells of about  $1 \times 10^7$ /ml were administered additionally one month later. Further, 1 ml of cells of about  $1 \times 10^7$ /ml were administered additionally several times at an interval of a month, and after the reactivity between the immunized rat antibody and CF-1 cells was recognized, 1 ml of cells of  $1 \times 10^8$ /ml were administered as the final immunization. Three days after the final immunization, the rat was killed to remove spleen.

#### 30) 2) Cell Fusion

After the spleen removed from the rat was minced, splenic cells isolated were centrifuged, suspended in an IMDM medium (Boehringer-Mannheim Co.), and washed intensively. On the other hand, the cells obtained by cultured mouse myeloma cell line Sp2/0-Ag14 [Nature, 276, 269-270 (1978)] in an IMDM (Boehringer-Mannheim Co.) supplemented with 10 % fetal bovine serum (FBS; Sanko Junyaku) were washed in the above IMDM medium in the same manner, and  $1 \times 10^8$  thereof and  $2 \times 10^8$  of the above splenic cells were put into a centrifuge tube and mixed to perform cell fusion by polyethylene glycol 4000 (Nakarai Kagaku) according to an ordinary procedure [Clin. Exp. Immunol., 42, 458-462 (1980)].

Subsequently, the obtained fused cells were dispensed into a 96-well plate with an IMDM medium supplemented with 20 % FBS, and cultured in an incubator under the condition of 37 °C and 5 % CO<sub>2</sub>. They were replaced into a HAT selective medium gradually from the following day, and continued to be cultured.

After the start of the culture, the supernatants were replaced into a new HAT medium twice a week to continue the culture and maintain the proliferation.

Next, the obtained fused cells were cloned according to an ordinary procedure using limiting dilution analysis. Namely, only clones having strong binding properties to antigens were cloned according to an ordi-

nary procedure employing limiting dilution analysis by examining binding properties thereof to the antigens, utilizing antibodies in the supernatants of the above fused cells.

### 3) Screening

The screening of fused cells (hybridomas) was performed according to indirect fluorescent antibody technique using flow cytometry.

The screening of clones producing objective antibodies was performed using CF-1 cells as target cells. Namely, cells suspended in a reaction buffer (PBS supplemented with 2 % FBS and 0.02 %  $\text{NaN}_3$ ) were centrifuged and recovered as pellets, then suspended in 100  $\mu\text{l}$  of the hybridoma culture supernatants (about  $1 \times 10^6/100 \mu\text{l}$ ) and reacted at 4 °C for 1 hour. After they were washed with the above buffer once, an FITC-labelled goat anti-rat IgG (FC) antibody (Chemicon) was added thereto and incubated for 1 hour. After they were washed once, they were analyzed according to flow cytometry (FACScan, Becton Dickinson).

### 4) Purification of Antibodies

The fused cells screened in the manner of the above 3) were cultured according to an ordinary procedure, and antibodies produced in the supernatants were separated according to an ordinary procedure, and purified.

Namely, hybridomas were recovered from wells with high antibody titers to the antigens, spread in a tissue culture plastic dish (Corning Co.), cultured under the condition of 5 %  $\text{CO}_2$  and 37 °C, proliferated, and purified according to an ordinary procedure to obtain monoclonal antibodies GSPST-1 and BMAP-1.

Regarding GSPST-1, obtained cells were injected into the abdominal cavity of a BALB/cAJc1-nu nude mouse (8-week-old, male, Nippon Kurea). Produced ascite was recovered after 10-14 days, salted out with 33 % ammonium sulfate, and dialyzed with PBS. Regarding the BMAP-1 antibody, it was cultured in a large scale in an Iscove's modified MEM medium supplemented with 10 % FBS, and the supernatants were concentrated, salted out with 33 % ammonium sulfate, dialyzed with PBS, purified again by means of a protein A column kit (Amersham), and dialyzed with PBS. Incidentally, in the above Example was described the case in which the CF-1 cells were used as antigens for immunization; however, it is possible to establish a monoclonal antibody in the same manner also in case of using other stromal cells having potency supportive of hematopoietic stem cells, and the present invention is not restricted to the above monoclonal antibodies but includes all monoclonal antibodies having the same characteristics and all hybridomas producing the monoclonal antibodies.

A hybridoma producing the monoclonal antibody BMAP-1 of the present invention is a novel fused cell prepared from a Wistar Imamichi rat splenic cell and a mouse myeloma cell line SP2/0-Ag14 as parent cells, and was

deposited on 9 August, 1993, under the name of BMAP-1 (rat mouse hybridoma) with the accession number of FERM BP-4382, at National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology in Japan [address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki 305, Japan], international depositary authority according to Budapest Treaty on the international recognition of the deposit of microorganisms for the purpose of patent procedures.

### 5) Properties of Antibodies

#### (i) Reactivity of Antibodies

#### (Reactivity to CF-1 Cells)

The results of examining the reactivity of the obtained monoclonal antibodies GSPST-1 and BMAP-1 to CF-1 cells according to immunofluorescence analysis are shown in Fig. 1 through Fig. 3. Here, Fig. 1 shows the results of analysis of the control in the absence of an antibody, Fig. 2 the results of analysis of the binding properties of GSPST-1 to CF-1 cells, and Fig. 3 the results of analysis of the binding properties of BMAP-1 to CF-1 cells. In the drawings, vertical axes show relative number of cells and transverse axes fluorescence intensity.

As is apparent from Fig. 1 through Fig. 3, it has been revealed that monoclonal antibodies GSPST-1 and BMAP-1 have properties binding to CF-1 cells and recognize surface antigens of CF-1 cells.

#### (Reactivity to Bone Marrow Cells)

Next, the results of analysis of the reactivity of GSPST-1 and BMAP-1 to normal bone marrow cells according to flow cytometry (FACScan, Becton Dickinson) are shown in Fig. 4 through Fig. 6. Here, Fig. 4 shows the results of analysis of the control in the absence of an antibody, Fig. 5 the results of analysis of the binding properties of GSPST-1 to bone marrow cells, and Fig. 6 the results of analysis of the binding properties of BMAP-1 to bone marrow cells. In the drawings, vertical axes show relative number of cells and transverse axes fluorescence intensity.

As is shown in Fig. 4 through Fig. 6, it has been revealed that GSPST-1 has not a property binding to bone marrow cells at all, and that BMAP-1 has a property binding to all bone marrow cells.

#### (Reactivity to Myelocytic Leukemic Cell Line (NFS-60))

The results of analysis of the the reactivity of GSPST-1 and BMAP-1 to NFS-60 cells [Proc. Natl. Acad. Sci. USA, 82, 6687-6691 (1985)] according to flow cytometry (FACScan, Becton Dickinson) are shown in Fig. 7 through Fig. 10. Here, Fig. 7 shows the results of analysis of the control in the absence of an antibody, Fig. 8 shows the results of analysis of the binding properties

of GSPST-1 to NFS-60 cells, Fig. 9 shows the results of analysis of the control using rat IgG1 on the market (Zymed) and Fig. 10 shows the results of analysis of the binding properties of BMAP-1 to NFS-60 cells. In the drawings, vertical axes show relative numbers of cells and transverse axes fluorescence intensity.

As is shown in Fig. 7 through Fig. 10, it has been revealed that GSPST-1 does not react with NFS-60 cells, and that BMAP-1 has a property binding to NFS-60 cells.

#### (Assay for BMAP-1 to Inhibit Proliferation of NFS-60 Cells)

The results of examining the action of BMAP-1 to NFS-60 cells in the presence of G-CSF 100 ng/ml and cyclohexyimide  $10^{-9}$  M according to the MTT assay method are shown in Fig. 11. Using culture plates with 96 wells, 10  $\mu$ l/well of BMAP-1 solution were added at concentrations of 0, 10, 100 ng/ml, and 1, 10, 100  $\mu$ g/ml to  $4 \times 10^3$ /well/100  $\mu$ l of NFS-60 cells, and two days after the numbers of living cells were measured according to the MTT method. It has been revealed as shown in Fig. 11 that the proliferation of NFS-60 cells is inhibited remarkably by BMAP-1.

#### (ii) Typing of Antibodies

Next, as a result of typing the subclass of IgG of the obtained monoclonal antibodies [using a rat Mono Ab-ID-Sp kit (Zymed) and a biotin-labelled mouse anti-rat IgG1 antibody (Zymed)], it has become apparent that GSPST-1 is IgG2a, and that BMAP-1 is IgG1.

#### (iii) Potency Inhibiting Bone Marrow Transplantation

Next, a test upon the inhibition of bone marrow transplantation was performed using these antibodies to examine characteristics thereof. The results are shown in Fig. 12 and Fig. 13. As is shown in Fig. 12 and Fig. 13, while BMAP-1 has the effect inhibiting the bone marrow transplantation, the effect has not been found in GSPST-1. Namely, the above results were obtained by administering  $1.0 \times 10^5$ /head of bone marrow cells and monoclonal antibodies to C57BL/6J mice, irradiated at a fatal dose of radiation (900 cGy), through the veins of tails, and counting the number of spleen colonies. Incidentally, "Non-treated" in Fig. 13 shows the case with no administration of bone marrow cells.

As is shown in Fig. 13, it has been confirmed that it is because BMAP-1 reacts with bone marrow cells to cause apoptosis that the monoclonal antibody inhibits transplantation completely in the test upon the inhibition of bone marrow transplantation. Namely, when a hybridoma producing BMAP-1 was administered into the abdominal cavity of a nude mouse, it died at the time when its ascite was stored in a small amount. In addition it has been revealed that all bone marrow cells died out according to the intravenous administration of 50  $\mu$ g/head BMAP-1 to a normal C57BL/6J mouse, and in

Fig. 14 is shown a microphotograph backing up the fact that bone marrow cells on 6 days after the intravenous administration of BMAP-1 died out. As is apparent from the microphotograph, it has been observed that not only lymphoid cells but also neutrophils, megakaryocytes, myeloblasts, myelocytes, mast cells, macrophages, monocytes and erythroblasts (so-called myeloid cells) died out. In addition, as a result of investigating the DNAs of the bone marrow cells of a mouse administered 30  $\mu$ g/head BMAP-1, apparently ladder formation has been observed as is shown in Fig. 15, and it has been revealed that the above reaction of BMAP-1 to bone marrow cells is due to apoptosis.

The Fc region of the IgG of the BMAP-1 antibody was digested with pepsin (Sigma) and purified by means of a GPC column as F(ab') $_2$ , and 33.5  $\mu$ g/head (corresponding to 50  $\mu$ g/head of the whole IgG) were administered to a C57BL/6J mouse intravenously; as a result of it, it was observed that bone marrow cells died out in the bone marrow. It has become apparent according to the above fact that neither antibody-dependent cell cytotoxicity nor complement-dependent cell cytotoxicity participates in the cell death of bone marrow cells by BMAP-1.

As an antigen causing apoptosis has been reported the Fas antigen of cell surface protein; regarding the Fas antigen, the expressions of mRNAs of it are recognized in the thymus, heart, liver, lungs and ovary, but few mRNAs of it are detected in the bone marrow [J. Immunol., 148, 1274-1279 (1992)], and hence it is apparent that antigens recognized by BMAP-1 are different from the conventionally known Fas antigen.

Furthermore, in order to make it clear whether an antigen recognized by BMAP-1 would be a TNF receptor or not, the function of BMAP-1 was investigated using L-929 cells reacting with TNF to cause cell death. The final concentrations of a mouse TNF  $\alpha$  (Genzyme) were 0, 1, 10, 100 pg/ml, 1, 10, 100 ng/ml, and 1  $\mu$ g/ml, and those of BMAP-1 were 0, 10, 100 pg/ml, 1, 10, 100 ng/ml, and 1, 10  $\mu$ g/ml, and the numbers of living cells of L-929 cells were measured according to the MTT method on the second day after the addition of the TNF  $\alpha$  and BMAP-1. As a result of it, as shown in Fig. 16, Fig. 17, while L-929 cells were reduced by TNF  $\alpha$  remarkably, BMAP-1 had no effect upon L-929 cells. Hence, it has become apparent that an antigen recognized by BMAP-1 is not a TNF receptor.

The results of investigating whether antigens recognized by BMAP-1 would be MHC class I antigens or not according to flow cytometry (FACScan, Becton Dickinson) are shown in Fig. 18 through Fig. 21. Here, Fig. 18 shows the results of analysis of the control using rat IgG1 (Zymed), Fig. 19 shows the results of analysis of the binding properties of the anti-mouse MHC class I antibody (rat IgG2a, BMA) to BWV1 cells (mouse lymphoma derived from BW5147 cells), Fig. 20 shows the results of analysis of the control using rat IgG1 (Zymed) and Fig. 21 shows the results of analysis of the binding properties of BMAP-1 to BWV1 cells. In the drawings, vertical axes

show relative numbers of cells and transverse axes fluorescence intensity. As a result, BMAP-1 did not recognize BWV1 cells but the MHC class I antibody reacted with BWV1 cells.

As described above, it has been confirmed experimentally that BMAP-1 has the function of causing apoptosis on myeloid cells; according to the present inventors' knowledge, no monoclonal antibody having the property of causing apoptosis on myeloid cells has been reported so far as described above, and hence monoclonal antibodies having such a function are novel ones found by the present inventors. And, since it is thought that the monoclonal antibodies of the present invention represented by BMAP-1 can cause death of myelocytic leukemic cells considered to be high in the expression of antigens thereof by utilizing the function of the apoptosis of the monoclonal antibody on bone marrow cells, the monoclonal antibody of the present invention having the property of causing apoptosis on myeloid cells is useful as medicine for myelocytic leukemia.

The monoclonal antibodies of the present invention have been described specifically according to the Example as above; as the monoclonal antibodies having the property of causing apoptosis on myeloid cells according to the present invention may be exemplified those mentioned as specific examples above, but they are not always restricted to them but include all monoclonal antibodies having the same characteristic and function prepared in the same manner, irrespective of the kind of antigens.

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#### Industrial Applicability

Since the monoclonal antibodies of the present invention are useful as antibodies recognizing and identifying antigens causing apoptosis on myeloid cells specifically and besides have the property of causing apoptosis on myeloid cells, they may be used as medicine useful in the field of remedies for myelocytic leukemia utilizing the property.

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#### Claims

1. A monoclonal antibody having the property of causing apoptosis on myeloid cells through binding to the myeloid cells.
2. Fragments of a monoclonal antibody having the property of causing apoptosis on myeloid cells through binding to the myeloid cells.
3. A hybridoma producing the monoclonal antibody according to Claim 1.

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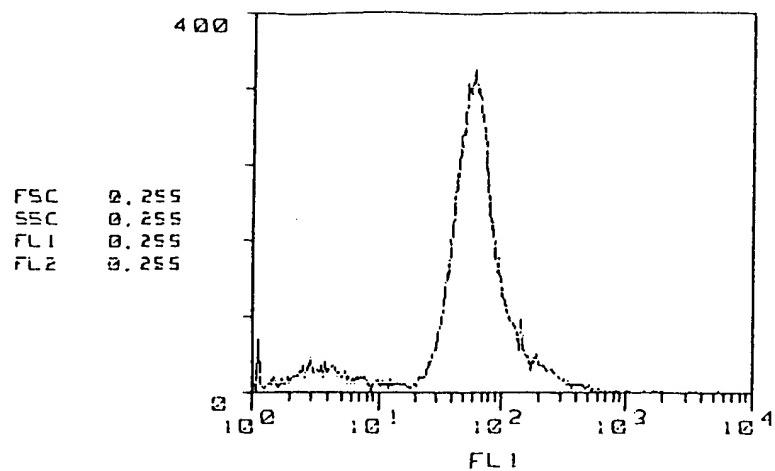


Fig. 1

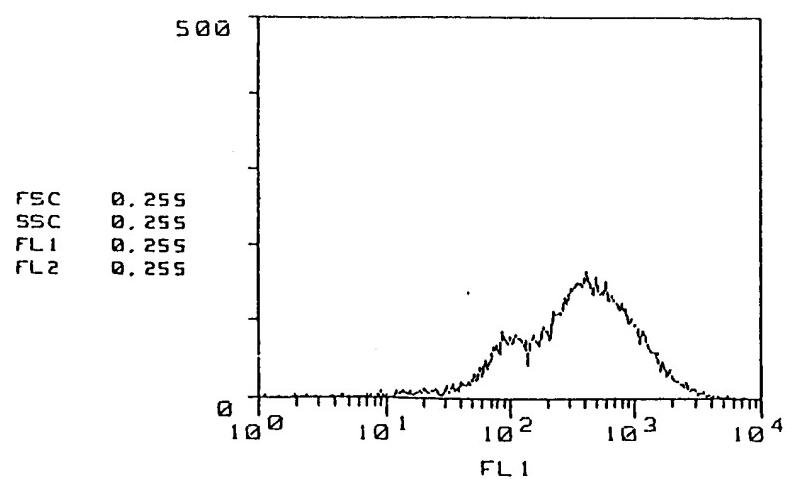


Fig. 2

**EP 0 721 015 A1**

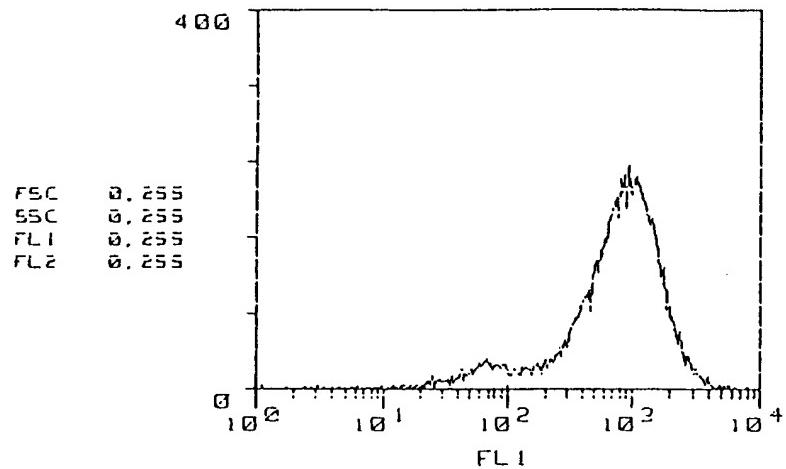


Fig. 3

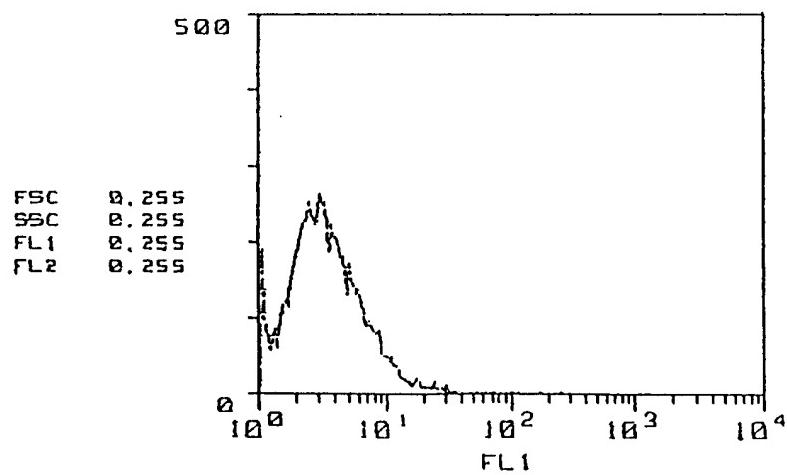


Fig. 4

**EP 0721 015 A1**

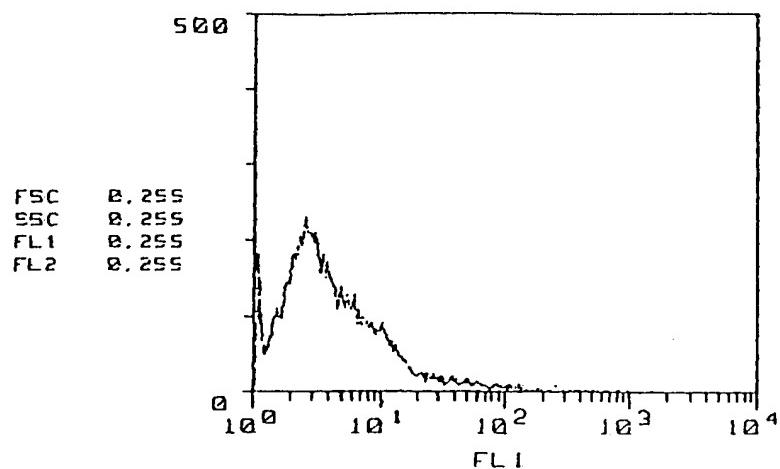


Fig. 5

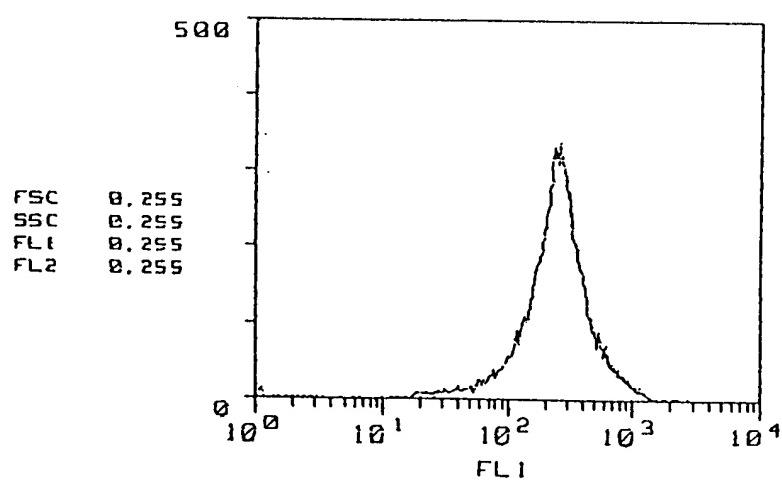


Fig. 6

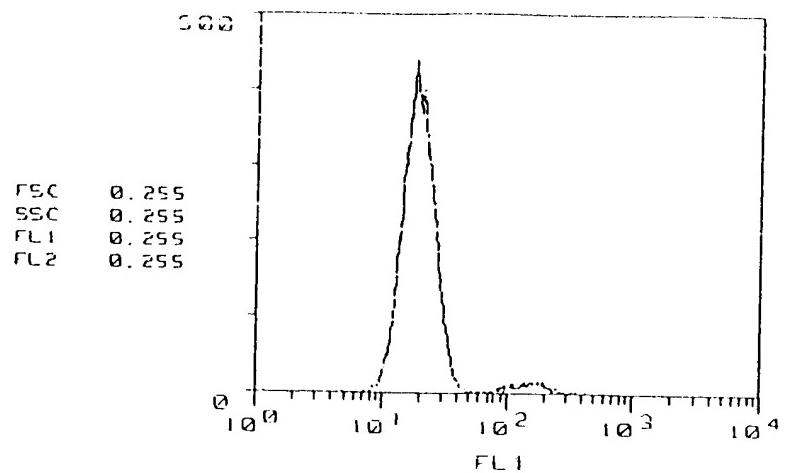


Fig. 7

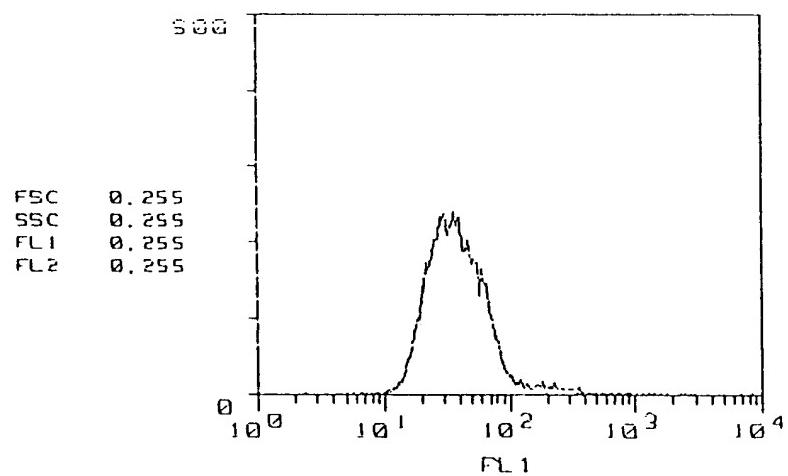


Fig. 8

**EP 0 721 015 A1**

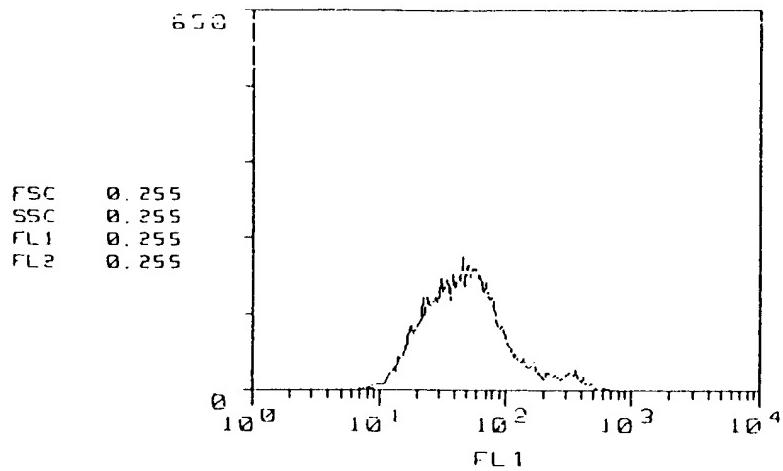


Fig. 9

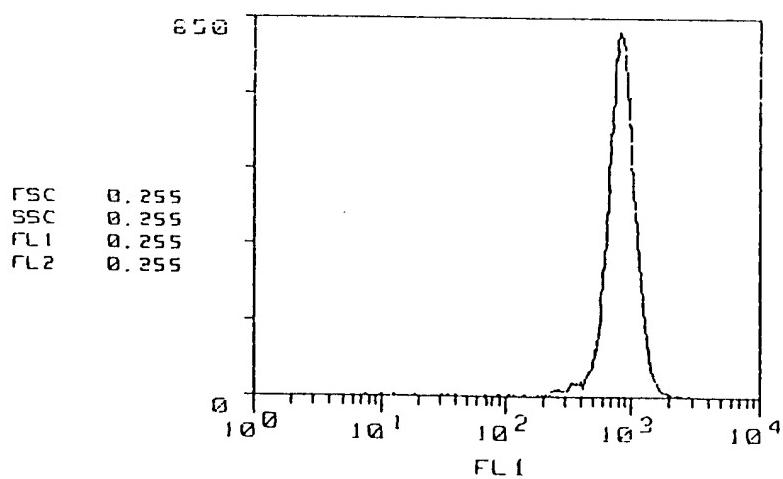
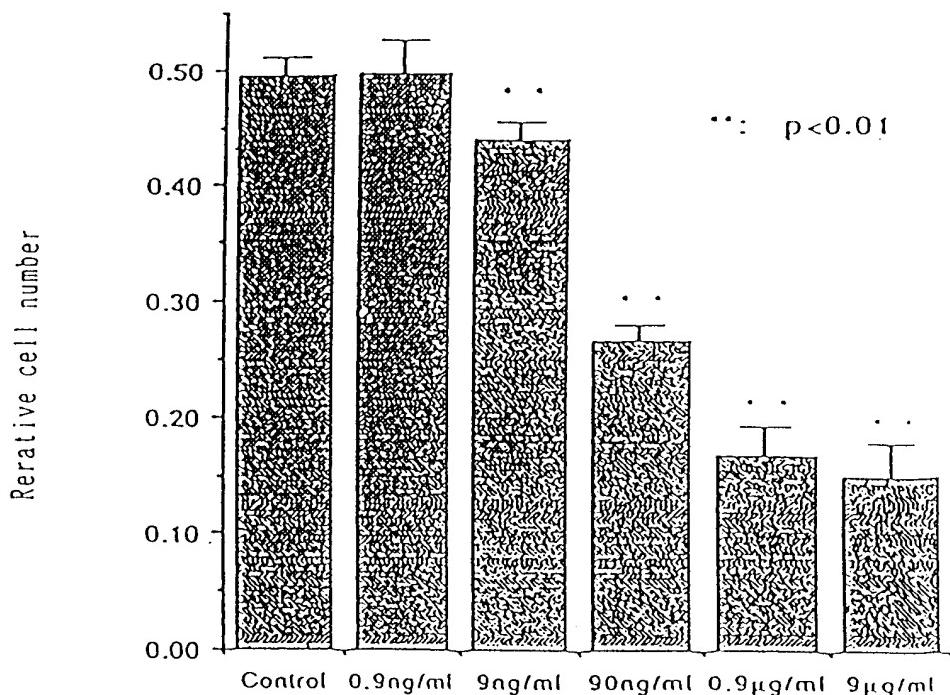


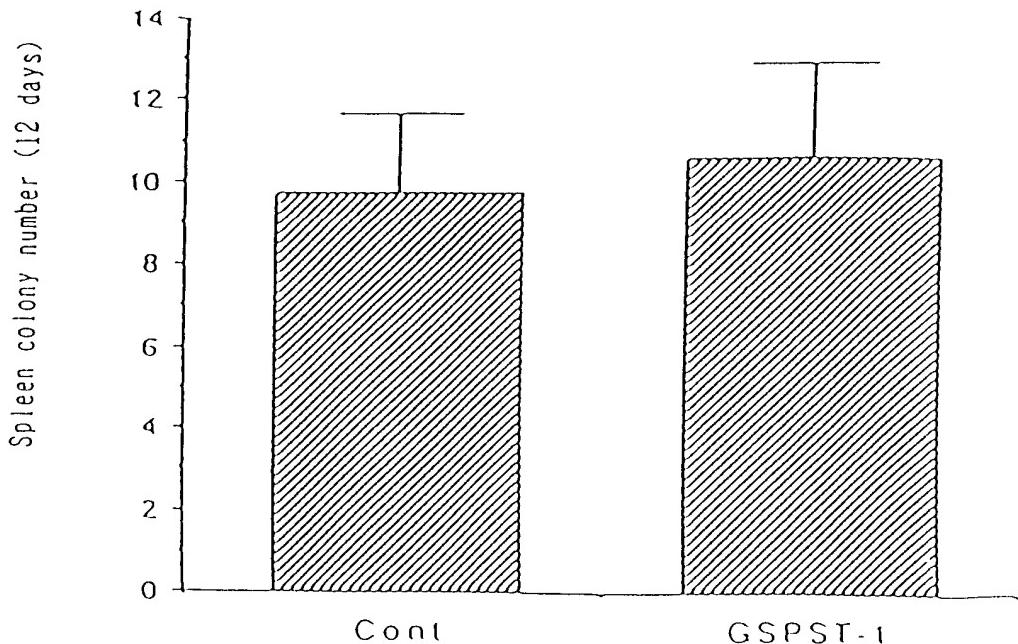
Fig. 10



Monoclonal Antibody (BMAP-1) Concentration

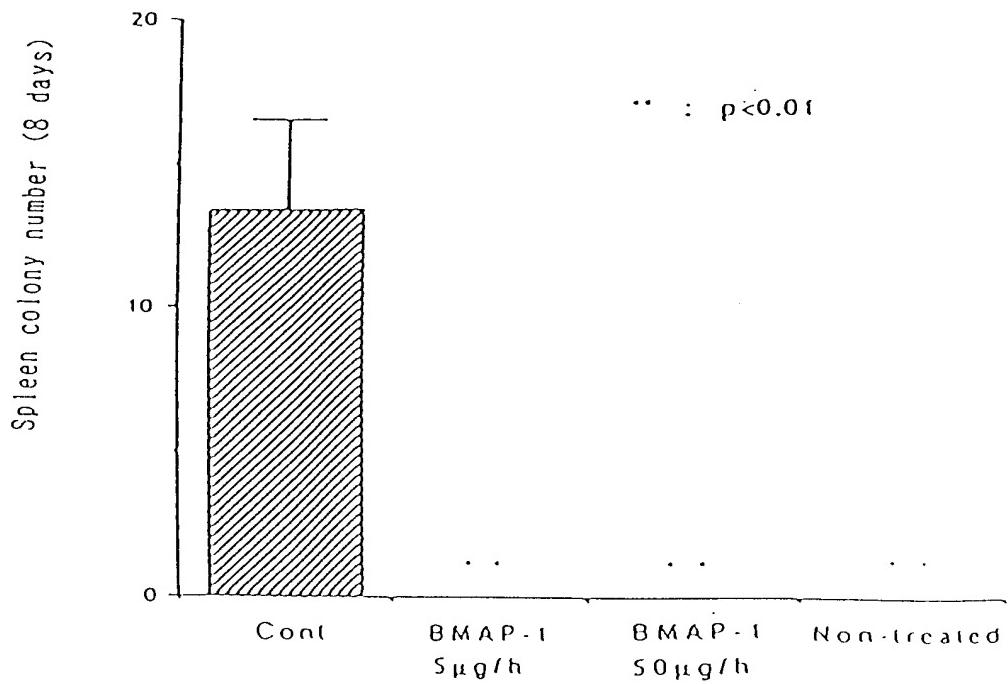
Assay for BMAP-1 to inhibit NFS-60 cell proliferation

Fig. 11



Monoclonal Antibody  
Assay for GSPST-1 to inhibit  
the bone marrow transplantation

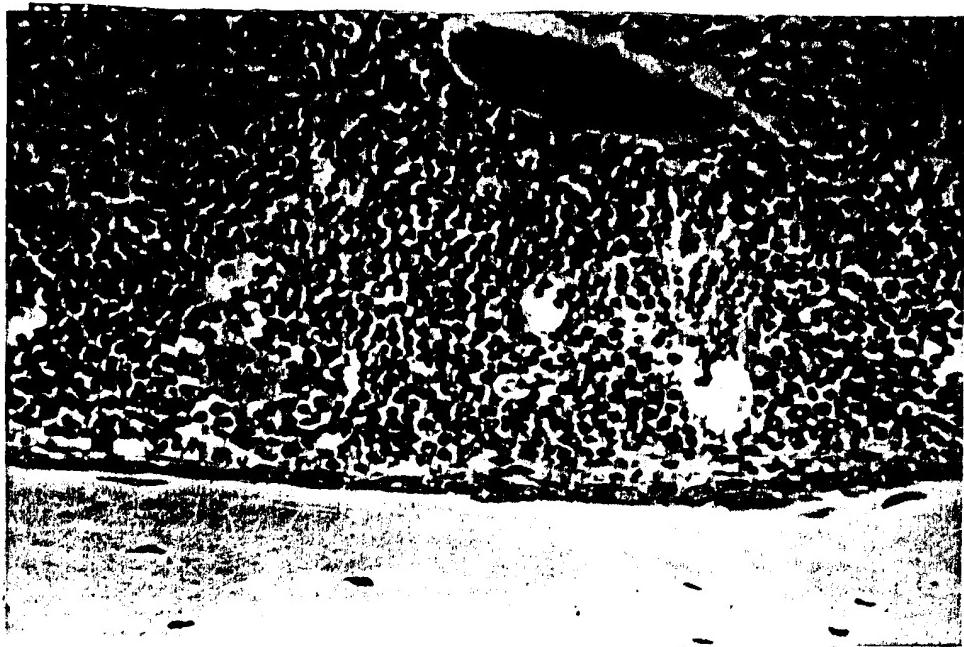
Fig. 12



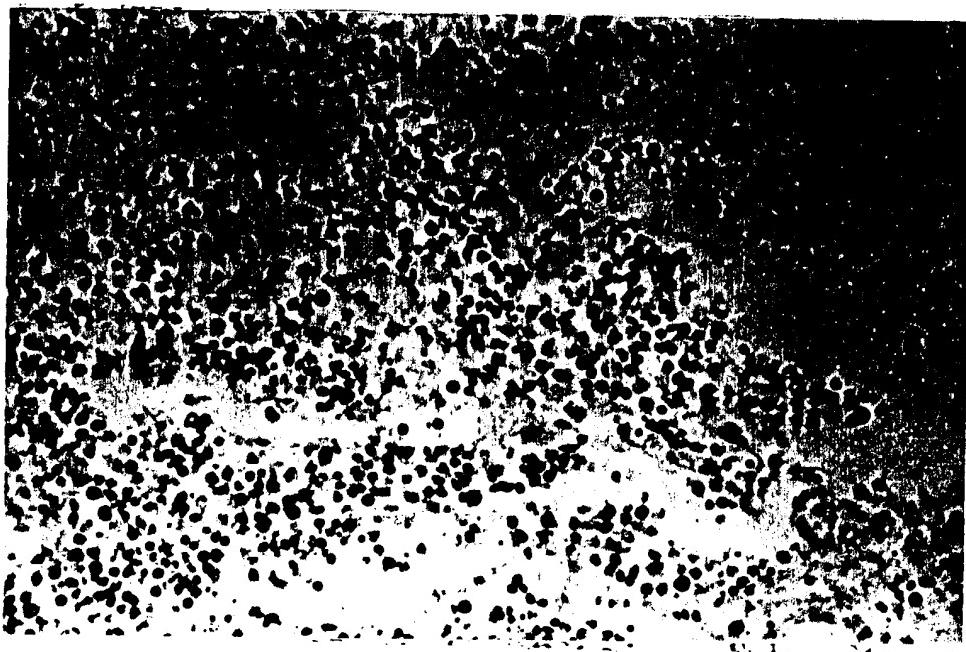
Monoclonal Antibody

Assay for BMAP-1 to inhibit  
the bone marrow transplantation

Fig. 13



(1)



(2)

Fig. 14

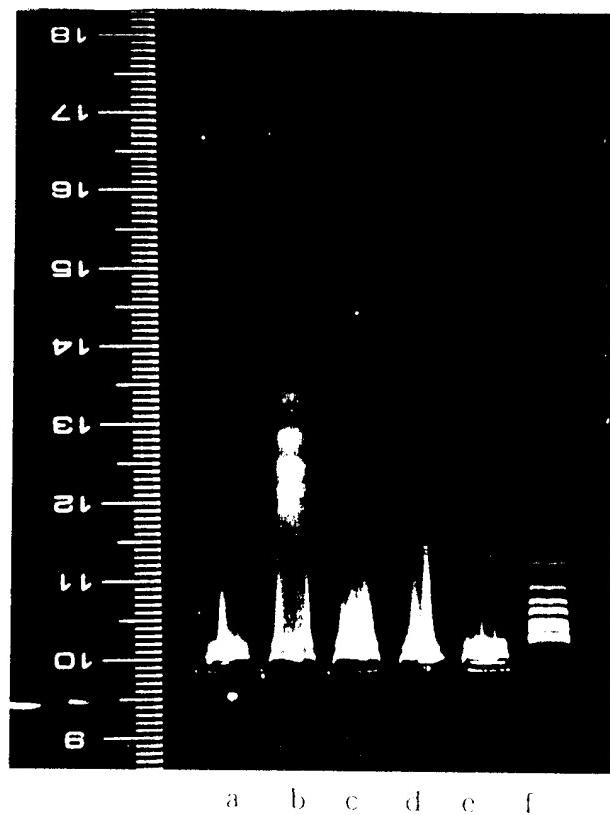
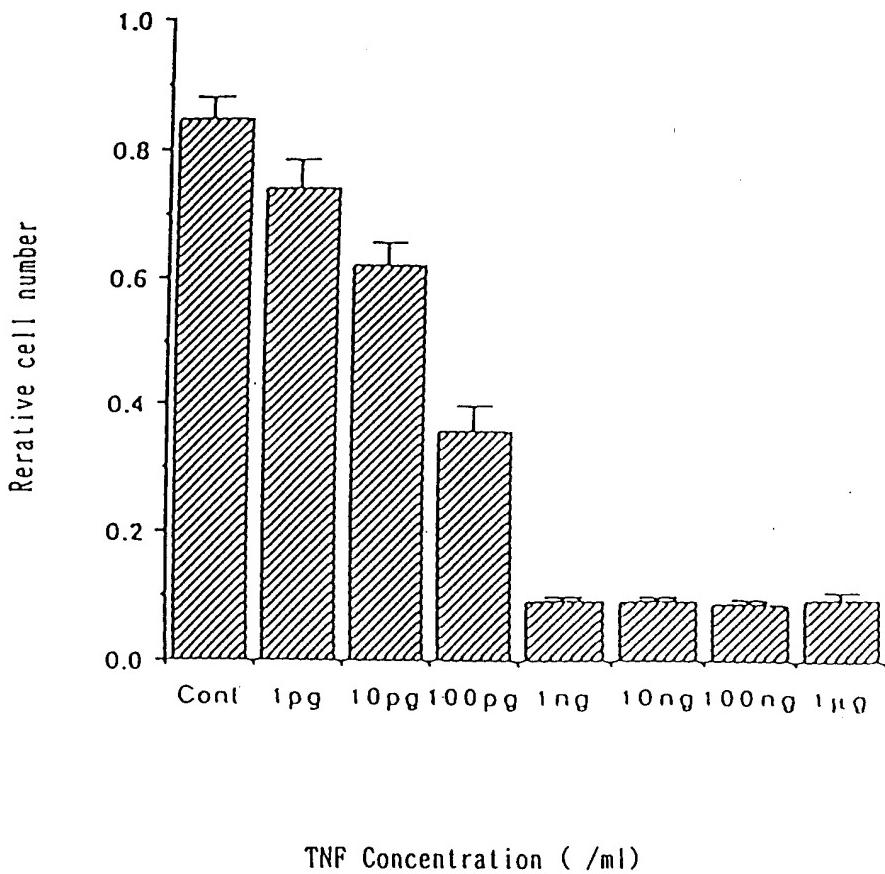
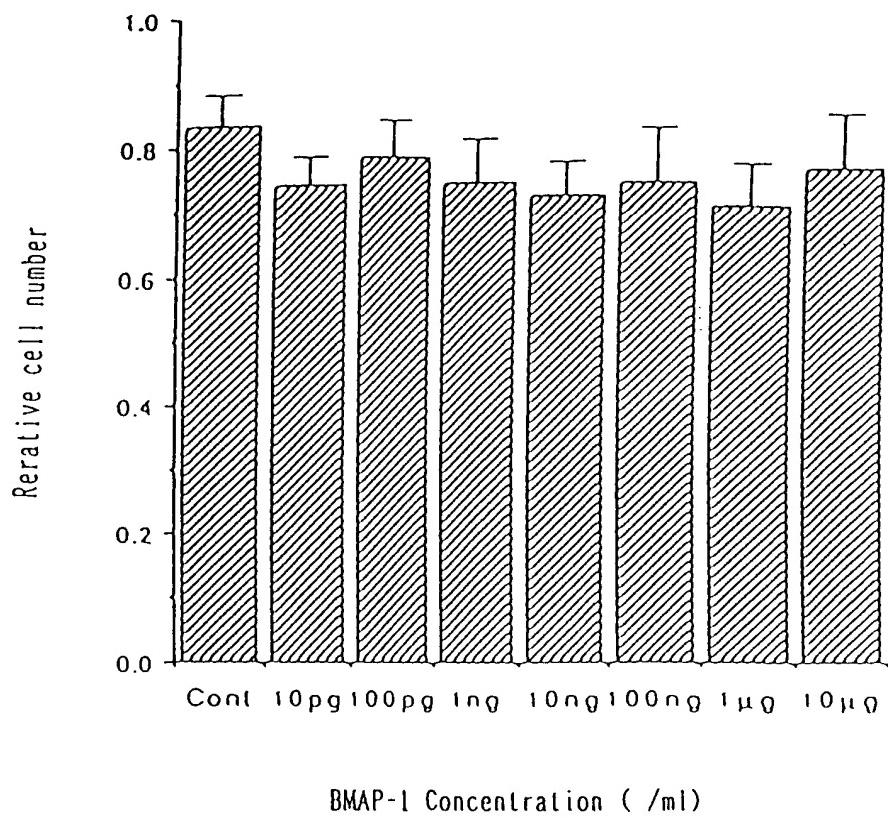


Fig. 15



Cytotoxicity assay using L-929 cells by TNF $\alpha$

Fig. 16



Cytotoxicity assay using L-929 cells by BMAP-1

Fig. 17

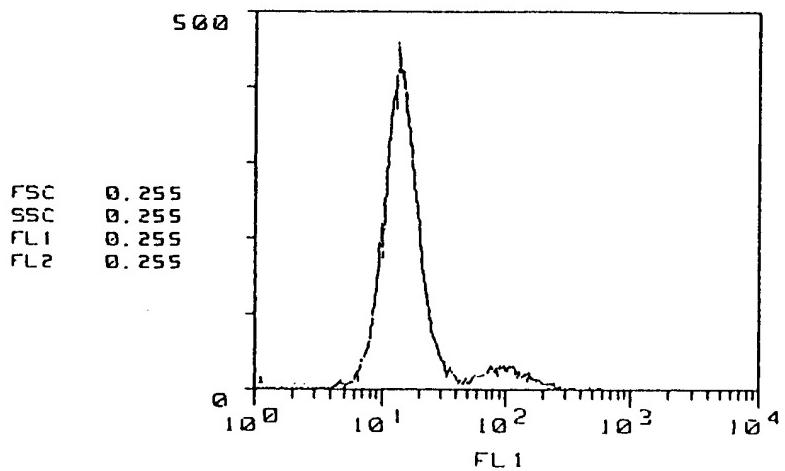


Fig. 18

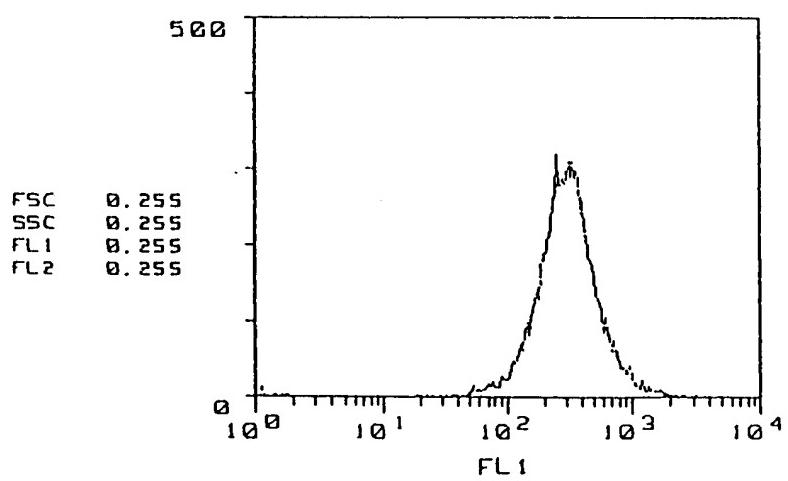


Fig. 19

**EP 0 721 015 A1**

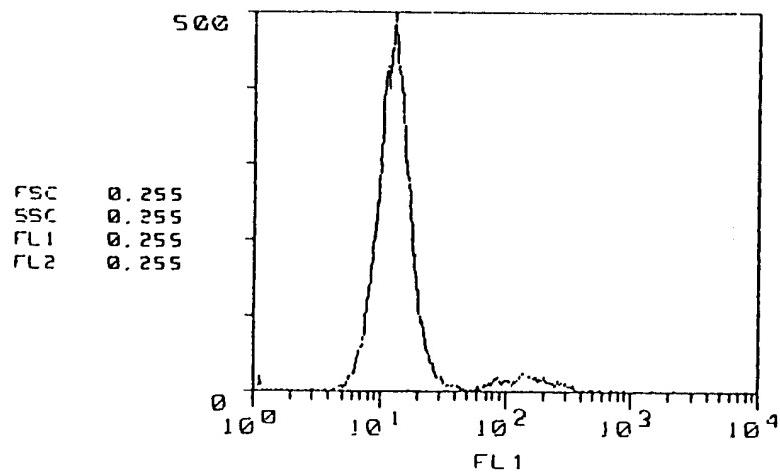


Fig. 20

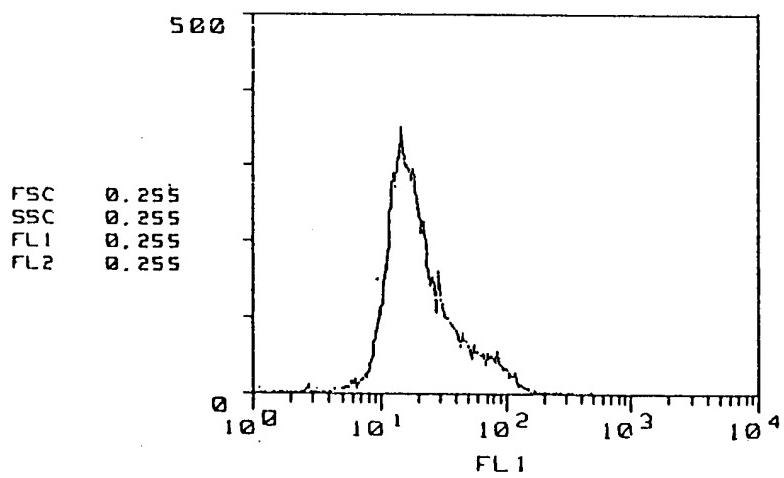


Fig. 21

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/JP94/01453

**A. CLASSIFICATION OF SUBJECT MATTER**  
 Int. C1<sup>6</sup> C12P21/08, C12N5/20, 15/06, C07K16/00, A61K39/395 //  
 (C12P21/08, C12R1:91)

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Int. C1<sup>5</sup> C12P21/08, C12N5/12-5/28, 15/02-15/90, C07K15/28,  
 A61K39/395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSIS PREVIEWS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Experimental Hematology, Vol. 16, No. 10, (1988), Stephen A. Cannistra, et al "Monocytes enhance gamma-interferon-induced inhibition of myeloid progenitor cell growth through secretion of tumor necrosis factor," P. 865-870	1-3
A	Neoplasma, Vol. 35, No. 5, (1988), B. Chorváth, et al "Immunocytometric characteristics of a monoclonal antibody (Bra 55) recognizing the leukocyte Common antigen (LCA)," P. 495-502	1-3

Further documents are listed in the continuation of Box C.

See patent family annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search  November 16, 1994 (16. 11. 94)	Date of mailing of the international search report  December 6, 1994 (06. 12. 94)
Name and mailing address of the ISA/  Japanese Patent Office  Facsimile No.	Authorized officer  Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)